—Mini Review—

Oocyte Cryopreservation

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Abstract: *Recent drastic advances of cryobiology and cryotechnology have enabled the cryopreservation of mammalian embryos and gametes. Improved vitrification methods have made it possible to preserve pre-fertilized oocytes with little loss of viability. Using a highly efficient vitrification method with ultra-rapid cooling (the Cryotop method), nearly 100% post-thaw survival rates have been obtained for human oocytes. These oocytes also have similar developmental ability* in vitro *after ICSI and IVC, and also to the term after ET. High post-thaw survival and pregnancy rates by the Cryotop method have been repeated at many IVF faculties throughout the world. The Cryotop method has contributed to the establishment of oocyte banks for unmarried cancer patients to preserve their fertility after chemo- and radio-therapy. Successful human oocyte banks have been established worldwide, resulting in the birth of more than 300 healthy babies so far.*

Key words: *Cryopreserved oocytes, Vitrification, IVF*

Introduction

The establishment and widespread application of an efficient and safe cryopreservation method for oocytes would not eliminate differences in reproductive flexibility between females and males, but could provide a solution to many specific problems and eventually reverse the actual trend of this unacceptable artificial widening of the gap between the two genders. Accordingly, this area deserves special attention, and should be regarded as more than just the subject of scientific ambition of a few, accidentally selected scientists, rather than an area that is benignly disregarded by the vast majority of reproductive specialists.

The need for change in the general attitude towards oocyte cryopreservation is even more justified by recent rapid advancement in technology offering the prospect of a real breakthrough and a definite solution in many important fields including the following ones.

1. Malignant diseases where systemic anticancer treatment is required [1]
2. Surgical procedures resulting in loss of ovarian function [2]
3. Treatment of patients with polycystic ovarian syndrome [3, 4]
4. Patients with ovary hyperstimulation syndrome [2]
5. Poor responders to ovarian stimulation [2]
6. Patients at risk of ovarian function loss through premature menopause [2]
7. In cases of male factor infertility or problems associated with difficulty of sperm collection, inadequate seminal samples or non-viable spermatozoa at the time of oocyte retrieval [2]
8. To overcome ethical concerns and legal restrictions associated with embryo cryopreservation in several countries [5]
9. Cryobanking of oocytes for young women who wish to delay motherhood for various reasons (career, lack of appropriate partner, etc.) [6]
10. Cryobanking of oocytes for egg donation programs or for research purposes [7]

Cryobiology of Oocyte Cryopreservation

Why was oocytes cryopreservation difficult? Some of the reasons including the size, shape, and cell number are quite obvious. It is well known that oocytes are the largest cells of the human body. In cryobiology, the size, or rather the mass is a decisive factor. Suspensions of somatic cell cultures can be cryopreserved with high efficiency and without any sophisticated approach by using simple media, a refrigerator and a deep freezer or liquid nitrogen. For even smaller bacteria and viruses, we meet frustrating evidence every day. They are present in almost every

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liquid nitrogen tank, and preserve their viability without any protection and in spite of our best efforts to eliminate them (although, in addition to size, some other factors, for example, their simple structure may also play a role in this resistance). In reproductive biology, the above mentioned differences between cryotolerance of spermatozoa and oocytes are at least partially attributable to their differences in volume. Quite controversially, the cumulative mass of cells decreases exponentially during the first week of embryo development, and at the expanded blastocyst stage it may become as low as 1/10 to 1/100 of that of the oocyte, with an obviously similar decrease of the water content. Although the solution accumulated in a blastocoel could be a potential source of damage either by ice crystal formation, or through the accumulation and slow dilution of toxic cryoprotectants [8–10], these phenomena cause more harm when they occur intracellularly in the oocyte.

Apart from the size, the shape of the oocyte is also the most unfortunate. The almost perfect sphere slows down formation of an equal distribution of any substance, including permeable cryoprotectants coming from outside or released from the oocyte. Accordingly, for a relatively long period of time a continuous concentration gradient from the periphery to the centre or *vice versa* exists, resulting in toxic damage in one part while providing less than optimal protection in the other. From this point of view, the change in shape caused by the osmotic effect at equilibration may offer some kind of benefit, but it may also contribute to the damage of the cytoskeleton (see below).

Another major factor is the lowest possible cell number. From this point of view the oocyte resembles a gambler who puts all his money on the very first bet: all or nothing. Multicellular embryos can survive and compensate for as much as 50% loss of their cells (and supposedly also some level of injury in the remaining ones) as demonstrated by biopsies, bisection of embryos, or just a less than optimal culture condition apart from the cryopreservation experience. The oocyte has only one chance, and there is no backup to regenerate from a serious injury. An extremely careful approach has to be taken to ensure the survival of the oocyte.

Apart from the factors listed above, there are still many other factors that contribute to the sensitivity of oocytes to cryoinjuries. There is chilling injury, that occurs at relatively high temperatures and induces irreversible damage of the cytoplasmic lipid droplets, lipid-rich cell membranes, and microtubules. This affects mostly the latter two structures in the human oocyte, as (in contrast, for example, to pigs) in humans cytoplasmic lipid droplets are less abundant. On the other hand, the membranes of human oocytes are extremely sensitive and rapidly undergo a transition from the liquid state to the gel state, an irreversible process that is detrimental to future development. For unknown reasons, just a step ahead, after fertilization the membranes of zygotes are much less sensitive to this type of injury [11].

The depolymerization of microtubules, misalignment of the chromosomes, and the possible increased risks of aneuploidy are frequently emphasized and have been shown in a wide experimental background [12–15]. However, human comparative examinations may not entirely confirm the seriousness of this problem [16], and the supposed beneficial effect of some agents (cytoskeleton relaxants or stabilizers) is not fully proven. Similar to somatic cell nuclear transfer, spindle reorganization may occur surprisingly efficiently, and the number of chromosomal abnormalities in children born after oocyte vitrification has not yet shown a significant increase.

A strange and not completely understood phenomenon is the change in cryosensitivity of oocytes during the maturation process. Although there is only a minimal difference between the size and shape, immature oocytes are usually more sensitive to cryopreservation than mature (MII phase) oocytes [11, 17, 18]. The contrary might be supposed, based on the known sensitivity of the meiotic spindle to chilling. More research is needed to understand the reasons for this difference, and the alteration of sensitivity of membranes may be one of the possible explanations.

Osmotic shock at equilibration may result in shrinking and deformation of the oocytes, supposedly damaging the cytoskeleton. However, the effect of other agents (for example pronase digestion of the zona pellucida) induces much more serious deformation, followed by surprisingly rapid recovery and the maintenance of developmental competence. On the other hand, the osmotic shock that may occur during dilution may result in extensive swelling, rupture of the membrane, lysis and immediate death of the oocytes.

Hardening of the zona pellucida, attributed by some authors to premature cortical granule release may cause decreased rates of fertilization [5].

Fracture is a common consequence of all cryopreservation procedures [19] and does not seem to occur more frequently in oocytes than in embryos. However, while the consequence for zona fracture may

be similar for both, embryos may survive some level of cell membrane damage, while for the oocyte, any injury at this level is evidently fatal.

Strategies towards Successful Oocyte Cryopreservation

Based on the points listed above, the principles of a successful cryopreservation strategy can be outlined. Although infrequent in biological study, theory is mostly justified by practice, although it should be confessed that the sequence of events was (as usual) inverted in cryopreservation as the empirically established methods were retrospectively supported by the subsequent detailed theoretical analyses of events.

Chilling injury

First, we need a method that minimizes chilling injury. So far, for mammalian embryos and oocytes two approaches have been successfully applied: the removal of the lipid droplets (by high-speed centrifugation and micromanipulation, although the latter step is not required with the use of some recent techniques) [20], and by increasing radically the cooling and warming rates to minimize the duration of exposure to the dangerous temperatures. As human oocytes contain relatively low amount of lipids, centrifugation does not significantly improve survival chances. On the other hand, all forms of traditional slow-rate freezing are obviously less appropriate for the purposes of oocyte cryopreservation than high-rate cooling vitrification strategies.

Cryoprotectants

The large cell mass and spherical shape of the oocyte necessitate the use of highly permeable cryoprotectants with low toxicity. As in many areas of vitrification in mammalian embryology, ethylene glycol is the candidate of choice for this purpose. According to earlier investigations in rabbits [21], the permeability of ethylene glycol is facilitated by dimethylsulphoxide (DMSO). Further studies have also demonstrated that DMSO may have a beneficial effect on spindle polymerization, consequently providing a protective effect at oocyte vitrification [2]. Although various proportions of DMSO and ethylene glycol were extensively tested for vitrification of bovine oocytes and embryos, the best results have always been achieved with a 1:1 mixture (unpublished data). To facilitate dehydration, thus decreasing the chances of intracellular ice formation, the addition of non-permeable cryoprotectants is also required. Various substances including polymers with low toxicity have been suggested for this purpose, however, the traditionally used sugars, i.e. sucrose or trehalose seem to be the most appropriate. Curiously, although trehalose has been reported many times to be superior to other sugars, in the past few years it has gradually disappeared from the list of frequently used cryoprotectants.

Concentration

In the past few years, two basically different strategies of equilibration before cooling have been applied. Martino *et al.* [22] suggested that dehydration may be even more important than cryoprotectant concentration for prevention of ice crystal formation, and suggested extremely short equilibrations for both the diluted and concentrated cryoprotectant solutions. Subsequently this strategy was successfully applied by many others to domestic animal oocytes and embryos. However, more recently another approach has received more attention, and seems to be more efficient for mammalian oocytes: an extended equilibration in a rather diluted first cryoprotectant solution, followed by a short, but slightly prolonged incubation in the second, relatively concentrated vitrification solution containing a non-permeable cryoprotectant [23–25]. Although the time of the exposure is significantly increased, the cumulative toxic effect (as a result of the lower concentration) may be the same or even lower, and the prolonged equilibration may ensure proper penetration of cryoprotectant providing appropriate protection to the entire oocyte.

Decreasing cryoprotectant concentration

The other way to minimize toxic and osmotic effects of cryoprotectants is to decrease the required concentration while maintaining the ice free solidification pattern. Currently the only practical way to achieve this goal is with an extreme increase in cooling rates. Among the various tools used for this purpose, electron microscopic grids, Cryoloops and Cryotops seem to be the the most appropriate, although very recently similar results were achieved with the open pulled straw technique [26]. Either directly by a higher rate of cooling, or indirectly by decreasing toxic and osmotic effects, Cryotop and Cryoloop vitrification with a mixture of relatively low concentrations of DMSO and ethylene glycol does not seem to cause serious anomalies in the spindle structure, and may ensure relatively high developmental rates [25, 27].

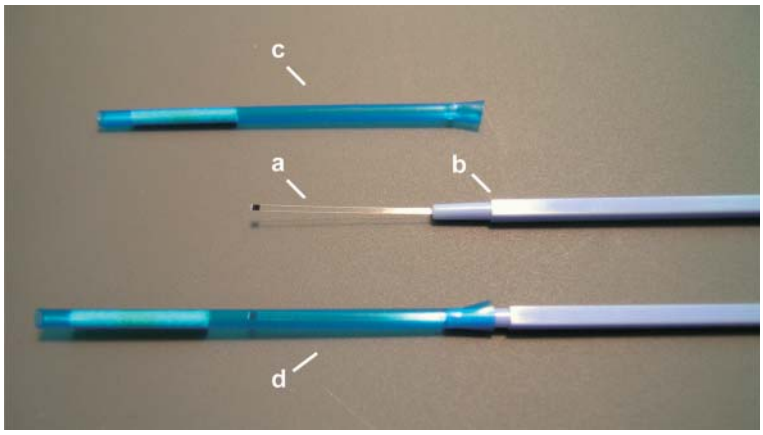

Fig. 1. The Cryotop vitrification tool. The polypropylene strip (a) is attached to a hard plastic handle (b). After vitrification, a hard plastic cover (c) is attached to protect the strip during storage in LN_2 (d).

As already mentioned, fracture damage is not specific to oocyte cryopreservation, although the consequences may be more detrimental. Fortunately, open vitrification systems have drastically reduced the occurrence of this type of damage. Retrospectively, it may be supposed that in a closed system, the extreme pressure changes caused by rapidly cooling or warming air bubbles induce dislocations in the partially solidified solution, and with a scissor-like effect cut the zona pellucida or the cell membranes. In open systems, such mechanical forces are almost completely voided. The extremely small volume of solutions used also minimizes the chance of fractures. Accordingly, this type of damage is almost entirely eliminated by utilizing ultra-rapid open vitrification systems.

Intracytoplasmic sperm injection (ICSI)

The problem of zona hardening and subsequent low level of fertilization has been eliminated entirely with the discovery and subsequent widespread application of intracytoplasmic sperm injection (ICSI). Although not an original objective, the application of ICSI after cryopreservation has contributed much to the increase of fertilization efficiency, and opened the gate for the widespread application of oocyte cryopreservation. However, very few human embryologists have had the courage to pursue the opportunities this approach seens to offer.

Clinical Results

The Cryotop method is now used in an increasing number of laboratories worldwide for oocyte vitrification as the best protocol for oocytes cryopreservation. Almost all of these laboratories have indicated survival, *in vitro* developmental and subsequent pregnancy rates much higher than those achieved previously with traditional freezing. However, since the technique has only been recently internationally acknowledged, published reports are still few in number. Here we summarize only documented achievements. It is strongly expected that reports with similar results will be published soon from other groups, as well, confirming the value of the technology for human oocyte cryopreservation.

Kuwayama *et al.* [6] reported high post-thaw survival and remarkable pregnancy rates after ET. Almost all oocytes survived vitrification and ICSI, and the cleavage rate did not differ from that of controls in our laboratory. When blastocysts were transfered, 45% of the vitrified oocytes developed to healthy babies. So far, the efforts of Kuwayama *et al.*, have resulted in the birth of more than 50 healthy babies after oocyte vitrification using the Cryotop method, and none of them have had any developmental abnormality. The Cryotop method has been adopted by many embryologists to answer patients' requirements world-wide. For example, Stehlik *et al.* [28] reported 97% post-thaw survival after vitrification of oocytes received in egg donations and reported the birth of the first baby in the USA, conceived from cryopreserved oocytes. Lucena *et al.* [2] reported 89.2% survival rates after Cryotop vitrification of oocytes, and a total of 56.5% pregnancy rates (13 of 23 patients) with an average of 4.63 embryos transferred to

patients. This Colombian group has also achieved the birth of a baby conceived after oocyte vitrification in South America. Ruvalcava *et al.* [29] have reported 401/445 (90.1%) survival and 34.1% pregnancy rates after Cryotop vitrification. Coba *et al.* (2007, submitted for publication) have vitrified a total of 225 MII oocytes. Two hundred seventeen (96.5%) survived cryopreservation, and 165 (76.0%) were normally fertilized after ICSI, a percentage which is not different from controls. Ninety-four percent of zygotes underwent cleavage on Day 2, and blastocyst per fertilized oocyte rates (22.4%) did not differ from the controls. Twenty-one embryo transfers were performed with vitrified oocytes resulting in 13 pregnancies (61.9% pregnancy and 37.2% implantation rates). Currently, 11 patients are pregnant (52.4%). Antinori *et al.* [30] also reported very high (99%) post-thaw survival and high pregnancy rates using the Cryotop method.

In total, more than 300 healthy babies have been obtained from vitrified oocytes using the Cryotop method so far.

Conclusion

According to the high number of ongoing pregnancies listed here and confirmed by personal communication with other laboratories, the number of babies born after Cryotop vitrification of oocytes may soon exceed the total number of babies born from other cryopreservation methods worldwide. All data obtained from different laboratories (including survival, fertilization, embryo development, and pregnancy rates) suggest that oocytes vitrified with this technology are highly viable, and their developmental competence is comparable with that of fresh oocytes. This increasing evidence proves that Cryotop vitrification may offer solutions for women with various fertility problems, and may contribute to compensation of the handicap of women from the standpoint of reproduction.

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